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The loss of vascular integrity and the consequent extravasation of intravascular fluid and blood cells into previously ischemic areas are initial events leading to tissue injury. The mechanism by which this damage occurs in the microvessels is unknown. The long-term goals of this project are to understand the second messenger system that controls microvascular angiogenesis so that better methods to prevent and control this tissue damage can be developed.

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Techniques that are used in this project include phase contrast and Nomarski light microscopy; electron microscopy; digital imaging fluorescence microscopy; immunocytochemistry for changes in cytoskeletal proteins (actin and vimentin); radiodetermination of cyclic AMP and cyclic GMP; determination of and changes in protein kinase C and protein kinase A activity; radiochemical determination of inositol triphosphate synthesis; and analysis of the protooncogenes c-myc, c-fos, c-sis and c-ras and their modulation during angiogenesis.

The progress report during the first year of the project covers the following main areas: development of better in vitro models of angiogenesis; studies of the role of cyclic AMP in angiogenesis; the effect of ischemia on angiogenesis; the effect of pH on ischemic angiogenesis; and the role of protein kinase C in angiogenesis. *Keywords*.

Adenosine phosphates, Guanosine monophosphate, (EDTA)

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**ROLE OF SECOND MESSENGERS IN ISCHEMIC TISSUE DAMAGE**

**ANNUAL REPORT: November 1, 1988 to October 31, 1989**

**R & T PROJECT CODE 441q806      Contract No. N0001489J1098**

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## 1. DEVELOPMENT OF IN VITRO MODELS OF ANGIOGENESIS

We have made substantial progress in improving the methods for the isolation and growth of skin microvascular endothelial cells. Using a Castroviejo keratotome to obtain thin sections of tissue, it is now possible to isolate microvascular endothelial cells without the need for the direct perfusion of the lumen of vessels with proteases. Instead, these thin sections can be floated on the digesting solution (0.3% trypsin-1% EDTA) for relatively short periods (45 minutes), and the cells released by pressure to the exposed cell surface. While we have used this technique mainly for the isolation of microvascular endothelial cells from the skin, we have also applied it in early studies on the isolation of microvascular endothelial cells from other tissues also damaged during ischemic injury. We have had success in isolating microvascular cells from both the heart and the kidney, and it is likely that this method will serve in the future as a general procedure to isolate endothelial cells from other organs as well. It was observed that the microvascular cells from these organs differ in their growth requirements, and the same conditions that have been developed for the growth and propagation of the skin microvascular endothelial cells do not permit the long-term survival of cells from other organs. As has been observed in organs in lower animals (e.g. rat), each organ has developed a unique vasculature to support its growth, and these observations now also apply to the human. It will however be possible to systematically decipher the conditions necessary for the maintenance of these cells in other organs in the way that has been used for cells from the skin now that a relatively simple method to obtain cells from these organs has been found.

## 2. EFFECT OF ISCHEMIA ON ENDOTHELIAL CELL MORPHOLOGY AND GROWTH

Exposure of microvascular endothelial cells to ischemic conditions in vitro has not shown the expected damage to the microvascular endothelial cells that we had anticipated. We have devised a system of tanks of oxygen, nitrogen, and CO<sub>2</sub> that permits the perfusion of culture dishes with any combination of oxygen, nitrogen, and CO<sub>2</sub> that may be required. Using this system, we have systematically studied the effects of time and oxygen tensions on the growth and morphology of both monolayer cultures of microvascular endothelial cells and on cells induced to form blood vessel by exposure to collagen gels.

When monolayer cultures of microvascular endothelial cells were grown in the presence of oxygen tensions ranging from 20% to 0%, and subsequently exposed to normal oxygen tensions, none of the morphologic changes observed in the microvasculature *in vivo* were observed *in vitro*. Surprisingly, not only were destructive changes not observed, but there was a mild stimulation in the growth of endothelial cells. The reason for this stimulation is presently unknown. These results have demonstrated a remarkable ability of the microvasculature to withstand prolonged periods of ischemia *in vitro* without significant damage.

In addition to the light microscopic observations, the ultrastructure of the cells was also studied to determine if damage to any of the organelles unique to the clotting process were damaged. We have paid particular interest to the endothelial-specific organelle the Weible-Palade body since this organelles appears to be the storage site for Factor VIII. Measurements of both the numbers, size, and distribution of these organelles did not change following extensive periods of both ischemia and reperfusion.

### 3. EFFECT OF ISCHEMIA AND pH ON ANGIOGENESIS

Since pH has been shown to affect the damage induced in tissues *in vivo*, the effect of both pH and ischemic conditions were measured. Cells exposed to pH 6.5 and ischemic conditions for periods up to 24 hours did not differ from controls grown under ischemic conditions at pH 7.2.

### 4. ROLE OF PROTEIN KINASE C IN MICROVASCULAR ANGIOGENESIS

Angiogenesis was induced in monolayer cultures by exposure of the apical surface of the culture to a collagen gel. Under these conditions endothelial cells reorganize into vessel-like structures. Phorbol myristate acetate (PMA) (10  $\mu$ g/ml) was used to stimulate and 1-(5-isoquinolinsulfonyl)-2-methyl piperazine (H-7) ( $10^{-6}$  M) to inhibit protein kinase C. Prior to stimulation with PMA cells were pulsed with  $^{32}$ phosphate and phosphorylated proteins were analyzed by two dimensional PAGE. PMA caused an increase in the rate and extent of new vessel growth. Addition of H-7 prior to PMA completely inhibited vessel formation and cells remained in a typical epithelioid monolayer. Removal of H-7 resulted in an immediate reorganization of the cells into vessels. Phosphate incorporation into soluble proteins was stimulated by TPA, and the incorporation was completely blocked by H-7.

These results demonstrated a close relationship between activation of protein kinase C, intracellular protein phosphorylation and new vessel formation. The ability to inhibit angiogenesis by inhibiting protein kinases may provide a new pharmacologic approach in the control of abnormal skin blood vessel growth.

These baseline studies will be duplicated in cells exposed to ischemic conditions in the subsequent years of the project.

##### 5. SECOND MESSENGERS AND ENDOTHELIAL CELL FUNCTION

We have demonstrated that cyclic AMP and calcium play major roles in skin microvascular physiology. The following is a manuscript draft that details our findings on the role of cyclic AMP in endothelial cell function.

## SUMMARY

The maintenance of the normal epithelioid morphology of human dermal microvascular endothelial cells (MEC) grown in vitro depends strongly the presence of factors which increase intracellular levels of cyclic AMP. Complete removal of dibutyrylcAMP-isobutylertethylzanthine (IMX) from the growth medium results in a progressive transition from an epithelioid to a spindle-shaped cell line. This transition cannot be reversed by the readdition of dibutyryl cAMP and IMX to the growth medium, or agonists which increase cAMP levels. Spindle-shaped MEC lose the ability to express Factor VIII rAG and Dr antigens and to bind PBML. Ultrastructural analysis of transitional cells and spindle-shaped cells show decreased numbers of Weibel-Palade bodies in transitional cells and a complete absence in spindle-shaped cells.

Interferon gamma alters several functional properties of both epithelioid and spindle shaped cells. In the absence of dibutyryl cAMP it increases the transition from epithelial to spindle-shaped cells. In the presence of cyclic AMP it increases the binding of PBMLs to each cell type and their endocytic activity.

These results suggest that cyclic AMP is a key second messenger in the maintenance of several important functions of microvascular endothelial cells. Factors that influence the levels of this messenger in vivo may be expected to influence the angiogenic and immunologic functions of the microvasculature.

## Introduction

Skin microvascular endothelial cells (MEC) participate actively and specifically in a number of important physiologic and pathologic processes including thromboresistance, tumor metastasis, tumor growth, wound repair and inflammation. In inflammation they respond to lymphokines by expressing class II MHC antigens, ICAM-1, and interact with lymphocytes (Bhan et al., 1982; Sobel et al., 1984; Geppert and Lipsky, 1985; Masuyama et al., 1985; Nickoloff, et al., Dustin).

In this study, in vitro cultures of dermal microvascular endothelial cells were used as a model to examine several factors that influence endothelial cell function. The mechanisms involved in the growth regulation of skin microvascular endothelial cell differ from those of the large blood vessel endothelium and microvascular cells in other tissues. (Auerbach et al., Charo et al.) Of particular importance is a strong dependence on exogenous dibutyryl cyclic AMP or agents that elevate intracellular levels of dibutyryl cAMP for the maintenance of optimal growth and typical endothelial cell morphology (Davison and Karasek. 1981). In the absence of cAMP, skin microvascular endothelial cells undergo an irreversible transition from an epitheloid morphology to that of a spindle-shaped cell (Davison and Karasek, 1981; Bensch et al., 1983).

To better understand the potential physiologic relevance of this transition, we have extended our initial observations on the role of cAMP on the differentiation and growth of MEC to its effects on the binding of peripheral blood leukocytes (PBML) to MEC, as it occurs in early inflammatory reactions, the expression of Dr Antigens, the lysosomal processing of foreign material, and the synthesis of Weibel-Palade bodies and Factor VIII.

Our results indicate that cAMP strongly influences each of these functions *in vitro* and that cAMP may be one of the main second messengers governing microvascular endothelial cell activity *in vivo*.

#### Materials and Methods

##### Isolation and Culture of Microvascular Endothelial Cells.

The cells lining the inner segment of the newborn foreskin dermis were isolated as described elsewhere (Kramer et al. 1980). Briefly, the inner segments were cut into 5 mm squares and incubated at 37°C for 45 minutes in a buffer solution containing 0.3% trypsin and 1.0% EDTA (Gibco, Grand Island, NY). The fragments were then rinsed in saline (0.9% NaCl), the epidermis removed, and the endothelial cells expressed from the dermis into Iscove's Medium (Gibco, Grand Island, NY) containing 2% pooled maternal serum,  $3.3 \times 10^{-5}$  M isobutylmethylxanthine (IMX) and  $5 \times 10^{-4}$  M dibutyryl-cAMP, antibiotics (penicillin, 200 units/ml; streptomycin, 200 units/ml and gentamicin, 50 mg/ml) using a blunt No. 10 scalpel blade. The cells were collected by centrifugation (800 x g for 1 minute) and resuspended in the above described medium (complete Iscove's Medium, CIM). All of the cells used in the described experiments were in their third or fourth passage of subculture. Cells were plated onto gelatin-coated 4-well (16mm) dishes, at approximately  $4 \times 10^4$  cells per well, or onto 35mm dishes (Lux, Thousand Oaks, CA) at approximately  $2 \times 10^5$  cells per well and the medium replaced weekly.

### Effect of cAMP and IMX on MEC Cultures

The effect of varying concentrations of dibutyryl cAMP and IMX on the growth and morphological characteristics of MEC cultures was evaluated on uniform populations of MEC plated onto 35mm dishes and at 80% confluence. Dibutyryl cAMP and IMX were added to the culture medium at 1/2 to 1/10 of the standard concentration ( $5 \times 10^{-4}$ ). As controls, MEC were grown in Iscoves Medium without dibutyryl cAMP and IMX and in Medium containing standard concentrations of dibutyryl cAMP and IMX. The cells were evaluated daily for morphological changes, estimation of confluence and changes in endothelial cell shape for a period of three weeks.

### Effect of Interferon-gamma on MEC Cultures

Transition of MEC from epithelial into spindle-shaped cells was analyzed in cultures grown on 35mm dishes in the presence of CIM with recombinant interferon-gamma (Biogen Corp., Cambridge, MA) (300 units/ml). After three days of incubation, the medium was removed and new medium without dibutyryl cAMP-IMX was added. Changes in morphology of MEC were followed by phase microscopy.

### Studies on DR-associated Antigen and Factor VIII and Expression in Cultures of Epithelial and Spindle-Shaped MEC

The expression of class II histocompatibility antigens in control and interferon-gamma treated MEC was analyzed using an anti-DR monoclonal antibody to the DR-associated antigen (Becton-Dickinson, Oxnard, CA) at a working dilution of 1/500; similarly, the expression of Factor VIII by these cells was studied with a monoclonal antibody against Factor VIIIr.Ag (Dako, Santa Barbara, CA) at a working dilution of 1/100.

For these analyses, endothelial cell cultures were fixed with cold methanol for ten minutes at -17°C, rehydrated with phosphate-saline buffer solution and exposed to the monoclonal antibody for one hour at room temperature. Antibody immunoreactivity was assayed as described before (Tuder, et al., 1987) using the avidin-biotin system (Vector, Burlingame, CA) and diaminobenzidine (Polysciences, Warrington, PA) as chromogen. The final reaction product was enhanced with 0.16% NiCl<sub>2</sub> which was added to the diaminobenzidine solution.

Peripheral Blood Mononuclear Leukocyte (PBML) Binding to MEC Cultures

PBML were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. The PBML were washed twice in RPMI 1640 (Gibco, Grand Island, NY) containing 10% newborn calf serum. 1.5 x 10<sup>6</sup> PBML were added to each of the duplicate wells containing spindle-shaped MEC that had been exposed to interferon-gamma (IFN-gamma) for three days. Controls consisted of duplicate cultures of similar population density of MEC previously not exposed to IFN-gamma. The dishes were then incubated for one hour at 37°C in a humidified atmosphere of 6% CO<sub>2</sub> and 94% air. At the end of the incubation period, the unbound PBML were removed by repeated rinsing with RPMI 1640 with 10% newborn calf serum. The cells were then fixed and stained with 1% methylene blue and 1% acridine for 5 minutes. The ratio of the number of bound PBML per normal-shaped MEC and spindle MEC was determined by counting bound cells in six microscopic fields.

### Electron Microscopy

MEC cultures grown in standard Iscoves Medium with IFN-gamma or in CIM without dibutyryl cAMP and IMX in the presence or absence of IFN-gamma were processed for electron microscopic examination as described elsewhere (Bensch et al., 1983). Ultrathin sections cut perpendicular and parallel to the surface of the plastic culture dish were examined in an Elmiskop 101 electron microscope.

### Phagocytosis Assays

Normal MEC were plated at a concentration sufficient to achieve 100% confluence ( $5 \times 10^4$  cells) onto 96-well plates (Nunc, Copenhagen, Denmark), the unattached cells were removed after one hour by rinsing with 0.9% saline and the plates overlaid with fresh standard medium. Half of the wells were exposed to IFN-gamma (300 u/ml) for three days. Thereafter 15-nm gold particles coated with rabbit immunoglobulin (E.Y. Laboratory, Burlingame, CA) in standard medium were added to the wells and incubated for one-half, two, three and four hours, respectively. The wells were rinsed twice with standard medium, the cells fixed with methanol at -17°C and exposed to porcine-anti rabbit antibody conjugated with peroxidase (Dako, Santa Barbara, CA). After 60 minutes incubation at room temperature, the wells were rinsed ten times with phosphate buffered saline containing 0.1% orthophenyl diamine (Sigma, St. Louis, MO) in citrate buffer and the reaction product was quantitated with an ELISA reader (BioRad, Richmond, CA). A total of four wells were analyzed for each one of the variables. Controls consisted of the cells in 16 wells that had not been exposed to the immunoglobulin-coated gold.

For electron microscopic examination MEC cultures were exposed to IFN-gamma as described above. On the third day of incubation, 20nm gold particles coated with goat anti-mouse antibody were added to the IFN-gamma

treated and to control cultures and incubated at 37°C for 2 and 12 hours, respectively. At the end of the incubation period, the cells were rinsed 5 times with normal saline (0.9%), fixed with 2% glutaraldehyde and processed for electron microscopic examination (Bensch et al., 1983). In an analogous study, MEC that had been left in CIM for two weeks which had led to transformation of all cells into the spindle type were assayed for their endocytotic activity as described above.

## RESULTS

### Cytology of Microvascular Endothelial Cells.

The MEC grown in medium containing  $5 \times 10^{-4}$  M dibutyryl cAMP and  $3.3 \times 10^{-5}$  M IMX exhibited morphological and growth characteristics typical of human dermal MEC as described previously (Bensch et al., 1983) (Figure 1A). Such cells, in their active phase of growth, develop an epithelial, polygonal appearance and grow in clusters having a cobblestone appearance. They express Factor VIII in a punctate pattern that varies in intensity. Electron microscopy confirmed that the normal MEC contain large numbers of Weibel-Palade bodies. Pinocytotic and, upon prolonged culture, large clear vesicles are conspicuous in these cells. The large vesicles often are of the multivesicular type or have a floccular content. Depending on the skin used, as much as 5-10% of the cell population may consist of melanocytic cells, the only other cell type present in these cultures. Morphologically the melanocytes are easily distinguishable from MEC by their characteristic long dendritic cytoplasmic processes and their usually very numerous melanosomes. Sections perpendicular to the culture dish reveal that melanocytic processes may extend a short distance underneath the thin outer edge of a MEC.

Evidence of direct passage of melanosomes from the melanocytes into MEC cytoplasm was never observed at these contact sites of melanocytes with MEC. Control cultures of pure melanocytes do not multiply in the absence of dibutyryl cycle AMP and no transition to a spindle shaped morphology of melanocyte cells occurs in the absence of cyclic AMP.

Effect of Decreased Concentrations of dibutyryl cAMP-IMX on MEC

Complete removal of dibutyryl cAMP-IMX from the medium leads to progressive changes in the morphological and functional properties of the endothelial cells (Fig. 1B). Small colonies of normal-shaped MEC separate from the remaining layer of confluent cells. In the periphery of these colonies the cells assume a slightly larger size, with polarization of their cytoplasm due to the formation of bipolar or tripolar cytoplasmic extensions. During the first 24 hours after the removal of cAMP-IMX, this process advances as more cells undergo a similar transition, resulting in progressively smaller colonies with the classic MEC appearance; at this time, the normal-shaped MEC are surrounded by cells in the process of spindling (transitional spindle MEC). Eventually, an entire cell culture consists of cells in the spindle-shaped configuration (Fig. 1C). The spindling process is irreversible after the formation of transitional MEC; i.e., replating these cells in new dishes in standard medium does not lead to a reversion to normal-shaped cells.

Lowering of the concentrations of cAMP and IMX compounds from 1/5 to 1/10 of their standard concentration in the medium led to a proportionally higher incidence of transitional and spindle cells. An analysis of such cells exposed to different concentrations of cAMP-IMX over a period of 3 weeks is shown in Fig. 2.

#### Effect of Interferon-gamma on MEC

The rate of transitional and spindle cell formation in MEC cultures exposed to IFN-gamma for three days in standard medium and in medium lacking dibutyryl cAMP-IMX was also determined. Hourly observations revealed that the transitional phase of MEC transformation is accelerated by IFN-gamma in comparison to control cells in cAMP-IMX-free medium. However, after approximately three hours both types of MEC cultures showed similar numbers of cells in the process of becoming the transitional shaped cells; thereafter, there were no differences in the rates of transition.

#### Functional Characterization of Normal- and Spindle-Shaped MEC

Normal MEC do not express DR-associated antigen; however exposure to IFN-gamma led to a uniform, strong expression of DR-antigen. Spindle and transitional cells present at the periphery of the colonies undergoing progressive spindle cell transformation do not exhibit immunoreactivity with the anti-DR antibody; this observation was made on spindle-shaped cells present in control cultures in standard medium as well as on those present in the cultures exposed to IFN-gamma (Fig. 3).

Similarly, the spindle MEC do not express Factor VIII though the normal-shaped MEC present in the same culture clearly exhibited cytoplasmic immunoreactivity, i.e., immunostaining was found only in cells having an epithelioid morphology and in some transitional cells.

Binding of PBML to normal MEC that had not been exposed to IFN-gamma is moderate at the end of one hour incubation (Fig. 4A). In the same cell cultures, binding to the spindle MEC was virtually absent and few PBML were found attached to transitional MEC that are present in the periphery of MEC colonies (Fig. 4B). IFN-gamma treated cultures showed a dramatic increase in PBML bound to MEC (Fig. 4C). In these cultures, even spindle MEC exhibited a significant number of bound PBML, although to a proportionally much lesser extent than were bound to the normal-shaped MEC. Fig. 5 shows a comparison of the ratio of PBML binding to different types of MEC with and without prior IFN-gamma exposure.

#### Ultrastructural Findings

The IFN-gamma treated epithelioid MEC were ultrastructurally similar to the control MEC. In cultures containing melanocytes numerous melanosomes were present singly or, more frequently, in large clusters in phagocytic and digestive vacuoles (Fig. 6). Sections perpendicular to the plane of a culture dish confirmed that the melanosomes were within MEC and not in melanocytic processes occasionally observed interdigitating with endothelial cell cytoplasm. Analysis of such areas of close contact between melanocytes and MEC did not reveal evidence of melanosomes being passed directly through cell membranes, thus suggesting that extracellular melanosomes had been engulfed by the endothelial cells with the phagocytic vacuoles having merged with the observed vesicular bodies which not infrequently contained partially digested melanosomes (Fig. 6B).

Fine structural examination confirmed the pronounced elongation and polarization of the cytoplasm of the spindle-shaped MEC. In these cells, Weibel-Palade bodies were markedly reduced in number, but those still present

had retained their characteristic elongate 2-4mm tubular structure indistinguishable from that found in control MEC (Fig. 7). Furthermore, there was a change in the distribution of the intermediate and microfilaments in the spindle cells, with presence of prominent subplasmalemmal bundles and a decrease of filaments in the perinuclear region. Also noted was a marked decrease in the number of pinocytotic vesicles in this cell type. Evidence of melanosome phagocytosis by this cells type, even after prolonged exposure to IFN-gamma, could not be found.

#### Endocytotic Activity of Normal and Spindle-shaped MEC

ELISA assays of the gammaglobulin bound to colloidal gold particles were used as a measure of endocytosis. These assays showed that the endocytotic activity of normal MEC was similar to that of IFN-gamma treated MEC. The assays revealed an increase in uptake over time up to three hours of incubation followed by a slight decrease at the four hour incubation point. In contrast, spindle-shaped cells showed considerably lower levels of endocytosis, a finding which was more striking with the cells not exposed to IFN-gamma (Fig. 8). Endocytosis of these cells approached the background level and it was significantly lower than that of normal MEC regardless of whether the spindle-shaped cells had or had not been exposed to IFN-gamma ( $t$  test,  $p < .001$ ).

Electron microscopic examination of INF-gamma treated MEC exposed to 20 nm gold particles coated with immunoglobulin for 2 or 12 hours showed that after 2 hours exposure, no major differences were noted in the number of endocytosed gold particles or the number of vacuoles containing clumped colloidal gold between control MEC and IFN-gamma treated MEC. After 12 hours of exposure, the IFN-gamma treated cells contained larger clumps of particles, as well as greater numbers of vacuoles containing colloidal gold than the control cells (Fig. 9).

## DISCUSSION

The results of these studies demonstrate that depletion of cAMP in cultured microvascular endothelial cells leads to a pronounced change in several functional properties of skin MEC. The cells lose their ability to grow as epitheloid colonies and become progressively elongate, leading to a uniform population of spindle-shaped cells. These changes cannot be reversed by readdition of cAMP to the culture medium or agonists that increase cellular levels of cAMP. This supports our previous observation that increased intracytoplasmic levels of cAMP induce higher plating efficiencies and maintain differentiation of MEC in culture (Davison et al., 1980; Bensch et al., 1983).

The morphological alterations of MEC brought about by a reduction of intracytoplasmic levels of cAMP affect the expression of DR-associated antigens after IFN-gamma exposure. The immunoreaction of the transitional- and the spindle-shaped MEC with a monoclonal antibody against DR-associated antigen was very weak or absent. Large vessel endothelial cells also elongate following treatment with IFN-gamma but, in contrast to MEC cultures, these cells continue to exhibit several IFN-gamma inducible functions including the expression of the DR-associated antigen (Pober et al., 1983; Groenewegen et al., 1985; Montesano et al., 1985; Masuyama et al., 1986; Stolpen et al., 1986). The observed induction of DR-associated antigen in MEC in vitro closely parallels the behavior of endothelial cells of microvessels in skin allograft rejection, in delayed-type hypersensitivity in skin test sites and in experimental encephalomyelitis (Bhan et al., 1982; Scheynius et al., 1982; Sobel et al., 1984). T lymphocyte proliferation and binding of these cells to endothelial cells in vitro has been shown to require the expression of class II MHC molecules by the vascular lining cells (Pober et al., 1983; Geppert and Lipsky, 1985; Masuyama et al., 1986).

Although significant binding of lymphocytes to large vein endothelial cells occurs only if cultures of such cells are exposed to IFN-gamma and thus induced to express class II MHC molecules (Masuyama et al., 1986), we have confirmed our previous observations that peripheral blood mononuclear lymphocytes (PBML) also bind to MEC in culture when they do not express DR-associated antigen molecules, at least as detectable by immunocytochemical methods (Nickoloff et al., 1988). Such binding of PBML to control cells could possibly still be triggered by minute amounts of mediators released by the PBML during the binding assay or by their stimulation by contaminating antigen presenting cells in the MEC cultures (Pober et al., 1983). It should be stressed at this point that relatively few transitional- and spindle-shaped MEC bound PBML under standard culture conditions; however, following exposure to IFN-gamma, PBML binding to these DR-associated antigen negative cells increased, but to a lesser extent than that observed with DR-associated antigen positive, normal-shaped MEC. Thus, a different class of membrane receptors may be involved in mediating, though less effectively, the binding of PBML to non-IFN-gamma-stimulated normal MEC and possibly also to the transitional- and spindle-shaped cells.

Our electron microscopic findings show that normal MEC exposed to IFN-gamma increase their pinocytotic and phagocytic activity. The IFN-gamma treated MEC contained large numbers of partially digested melanosomes which originated from melanocytes present in the cell culture system. This observation was strengthened by studies made on normal and spindle-shaped MEC after exposure to immunoglobulin-coated gold particles. IFN-gamma increased endocytosis in both spindle-shaped and epitheloid cells. However, the increase of endocytosis by epitheloid cells over control cells was minimal suggesting that under standard culture conditions, normal MEC are already maximally endocytotically active.

The observed ability of endothelial cells to ingest particulate matter and their ability to bind lymphocytes under stimulation by interferon gamma suggest that these cells may function as antigen presenting cells (Hirschberg et al., 1974; Pober et al., 1983; Geppert and Lipsky, 1985).

Details of the biochemical mechanisms by which cAMP maintains epitheloid morphology and promotes growth remain unknown. In other skin cells (keratinocytes, melanocytes) cAMP also stimulates growth while it simultaneously inhibits proliferation of fibroblasts (Friedman et al., 1982). Previous studies have shown that macrovascular endothelial cells in contrast to microvascular cells do not require cAMP for in vitro growth and that they continue to express functional properties in vitro in the absence of cAMP (Davison et al., 1981). The importance of cAMP for the in vivo response of vessels to injury and nearby inflammatory processes remains to be determined; levels of this cyclic nucleotide may control regulatory mechanism leading to the involution of microvessels when they are no longer required at a specific site, such as in a healing inflammatory focus. Interestingly, the speed of transformation of MEC after removal of cAMP from the culture medium suggests that major shifts in functional states of endothelial cells can occur very rapidly.

Under the experimental conditions used in this study, a proliferating spindle cell population eventually appears following a transitional phase of transformation of MEC after removal of cAMP-IMX from the medium. These fibroblast-like endothelial cells could easily be mistaken as an overgrowth of fibroblastic contaminants if they did not contain characteristic Weibel-Palade bodies, albeit present in much smaller than normal numbers. Similar shaped microvascular endothelial cells form after transformation of MEC with SV-40 virus (unpublished observations).

Though we do not know if such cells may eventually develop into a permanently transformed cell line and possibly acquire the capacity of producing tumors, the spindle-shaped MEC observed in this study resemble the spindle-shaped cells present in Kaposi's sarcoma. This peculiar, multifocal overgrowth arises in a setting of immunosuppression such as seen in AIDS and in transplant recipients and it has been assumed to be histogenetically related to lymphatic or blood vessel endothelial cells. (Rutgers et. al., 1986; Hashimoto et. al., 1987) Whether changes in the intracytoplasmic levels of cAMP in vivo are associated with the pathologic changes in this syndrome remains an interesting question.

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## LEGENDS

Fig. 1: Phase micrograph of endothelial cells in monolayer culture.

- A) Growth pattern in standard medium.
- B) Exposure for 2 weeks to standard medium with interferon-gamma causes moderate changes in cell shape such as slight cell elongation, i.e., "transitional" cells.
- C) Growth in standard medium without cyclic AMP leads to pronounced spindle cell formation within 2 weeks. Mag. X342

Fig. 2: Time Course of Epithelioid-Spindle Shape Transition

The time course of cell shape change into spindle cells is affected by the composition of the culture medium. All of the cells were grown in their respective medium for 3 weeks after which the further changes on number of spindle shaped cells were quantitated.

- A) Cells grown in standard medium which was changed weekly (closed triangles).
- B) Cell grown for 3 weeks without a medium change (zero point on the graph). 45% percent of the cells eventually become spindle-shaped (dotted square).
- C) Cell grown at 1/5 of the normal concentration of cyclic AMP (open squares) 80% of the cells are spindle-shaped.
- D) Cells grown in the absence of cAMP (closed squares) - 100% of cells became spindle-shaped.

Fig. 3: Effect of interferon-gamma on DR associated antigen in epithelioid and spindle-shaped cells

Cells had been growing in standard medium for 3 weeks before exposure to 300 u interferon-gamma/ml for 3 days. The center of the illustration shows a focus

of epithelioid cells surrounded by transitional and spindle-shaped cells. The dark staining of the epithelioid cells a result of their immunoreactivity with antibody to anti-DR associated antigen (peroxidase reaction). Note the absence of reactivity of the spindle and transitional cells. Mag. X200

Fig. 4: Binding of peripheral blood mononuclear leukocytes (PBML) to endothelial cells in monolayer culture. (See text for details.)

- A) Moderate numbers of PBML bind to cells grown in standard medium.
- B) In contrast, binding to spindle cells is virtually absent.
- C) Three days of exposure to medium with interferon-gamma leads to a dramatic increase in PBML binding to epithelioid cells and to a lesser degree also to the transitional and spindle cells.
- D) Higher magnification of transitional and spindle cells shown in micrograph

C. Mag. A,B,D, X350; C, X140.

Fig. 5: Ratio of number of bound peripheral blood leukocytes to IFN-gamma treated normal MEC (IFN-NL MEC), control normal MEC (CTL-NL MEC), IFN-gamma treated spindle MEC (IFN-spindle) and control spindle MEC (CTL-SPINDLE). For each of the experimental conditions 6 fields containing between 350 and 400 endothelial cells were averaged.

Fig. 6: Phagocytosis of melanosomes by endothelial cells

Endothelial cell cultures usually contain a small subpopulation of melanocytes. In interferon-gamma exposed cultures a striking increase in the number of ingested melanosomes in endothelial cells takes place. Fig. A shows an endothelial cell, cut parallel to the culture dish surface, which contains numerous vacuoles filled with melanosomes. These bodies undergo partial

breakdown in the digestive vacuoles, as shown in Fig. B. Note the Weibel-Palade bodies in this cell (arrowheads). Mag. X6,400 and X60,000.

Fig. 7: Electron micrograph of a spindle-shaped cell of a culture deprived of cyclic AMP (as shown in Fig. 1C)

Proliferating cells still retain certain features characteristic of endothelial cells, such as Weibel-Palade bodies (arrowheads). Mag. X25,000.

Fig. 8: Effect of interferon-gamma on endocytosis of epithelioid and spindle-shaped cells

Endocytosis of gamma globulin (globulin-coated gold particles) was quantitated by the Elisa assay (see text for details). Note the difference in uptake between normal and spindle-shaped control and interferon-gamma treated cells. Bars indicate the standard deviation from the mean of 16 assayed wells for each of the cell types.

Fig. 9: Electron micrograph of an endothelial cell exposed to gamma globulin-coated gold particles for 2-1/2 hours

The insert shows pinocytosis of such a particle at the cell surface facing the medium. Individual gold particles present in cytoplasmic vacuoles are indicated by the short arrows and a clump of these, in a multivesicular body, by the long arrow. The arrowhead points at a particle on the surface of the cell facing the plastic dish, possibly having been secreted by the cell. Mag. X36,000; Insert X54,000



FIG. 1

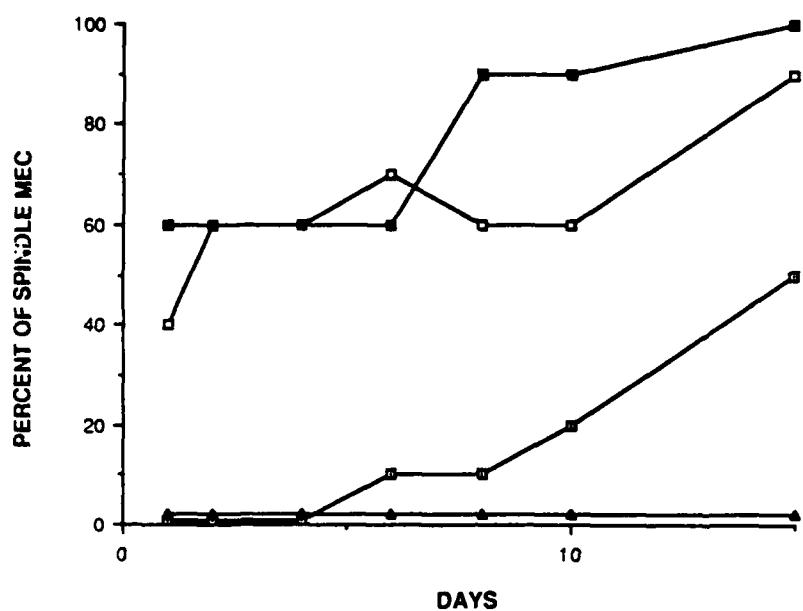


FIG. 2



FIG. 3

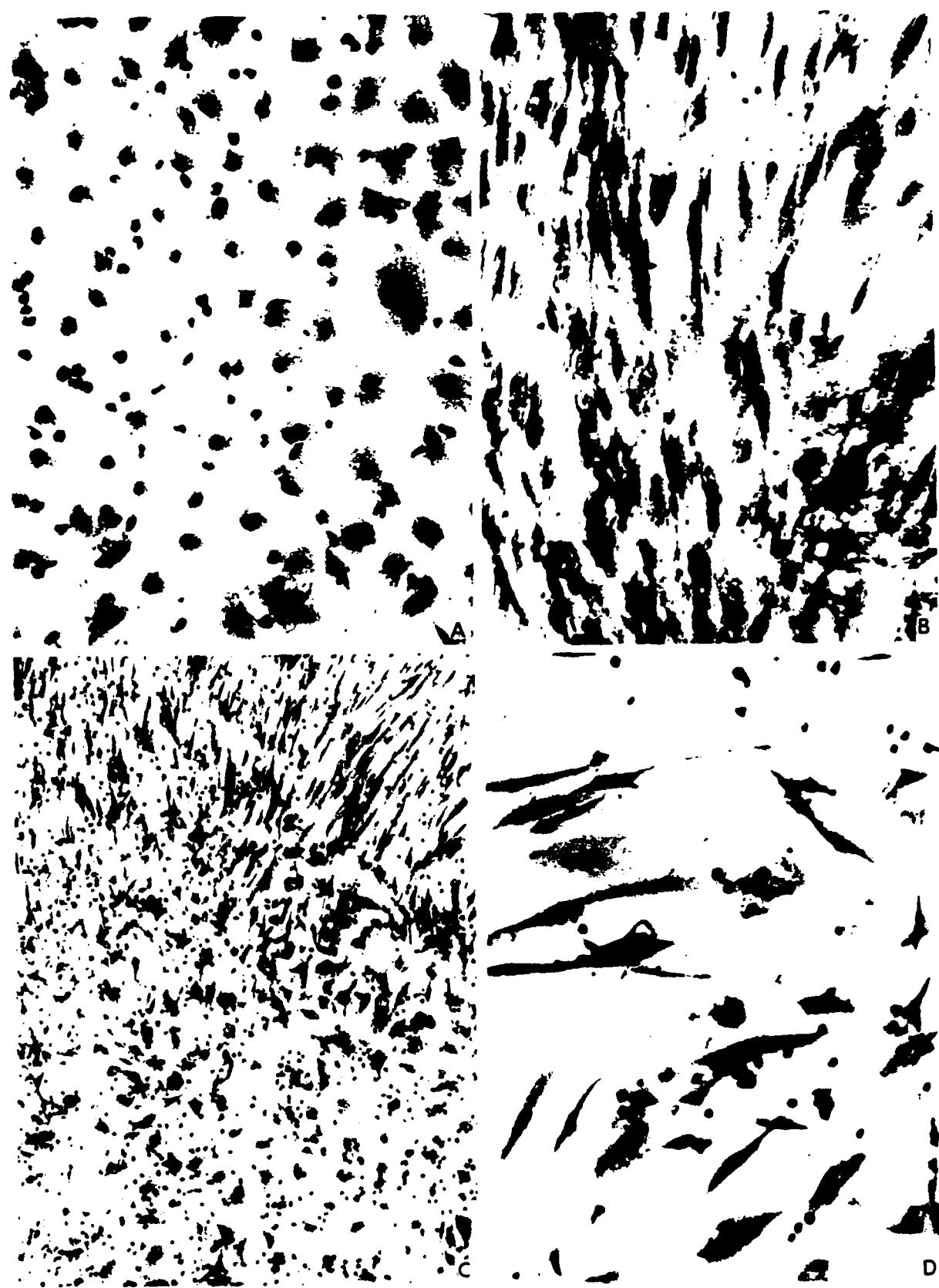


FIG. 4

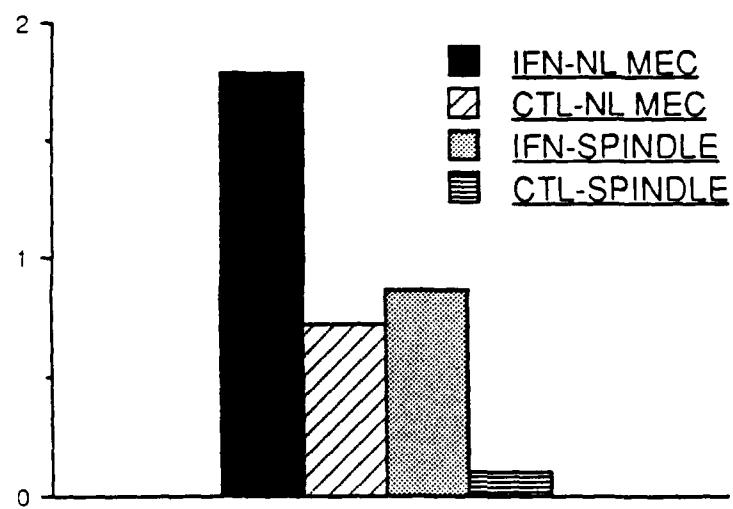


FIG. 5

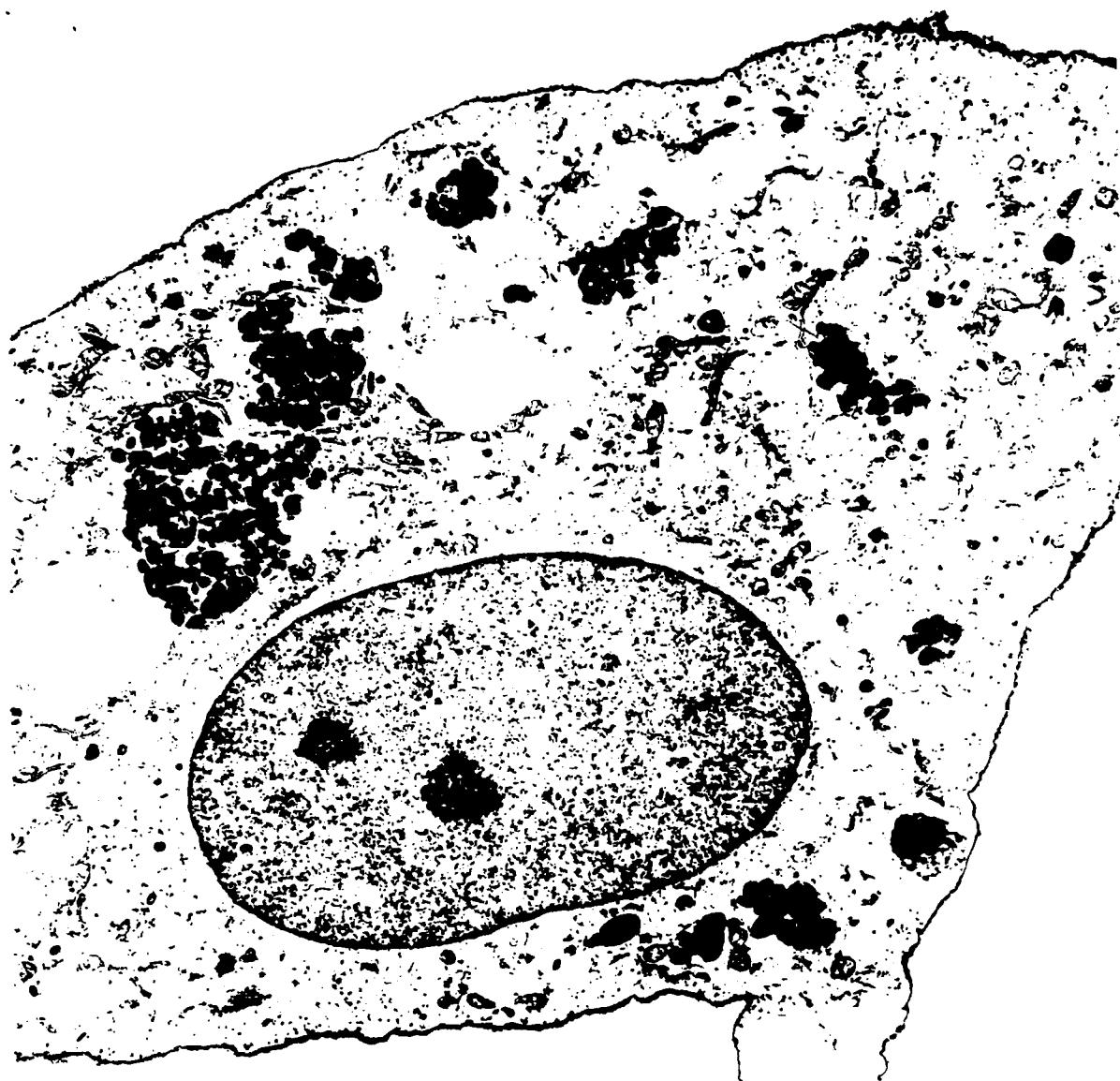


FIG. 6 A



FIG. 6 B



FIG 7

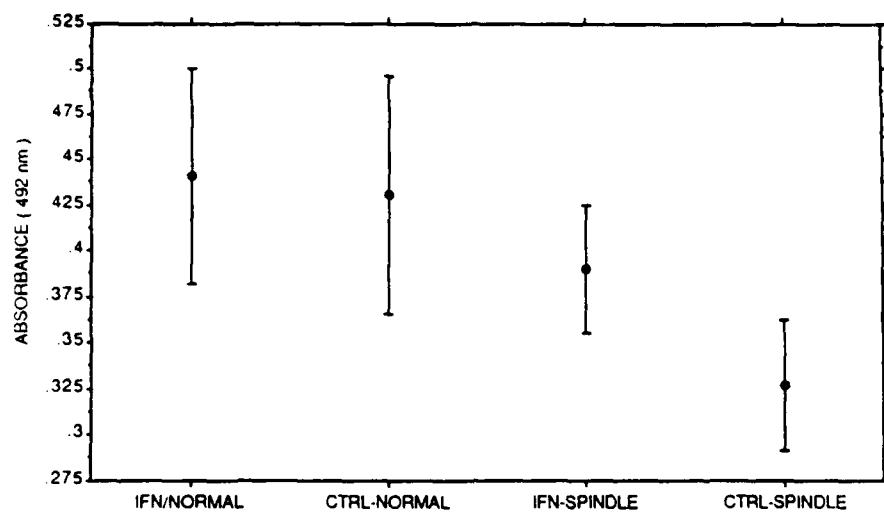


FIG. 8

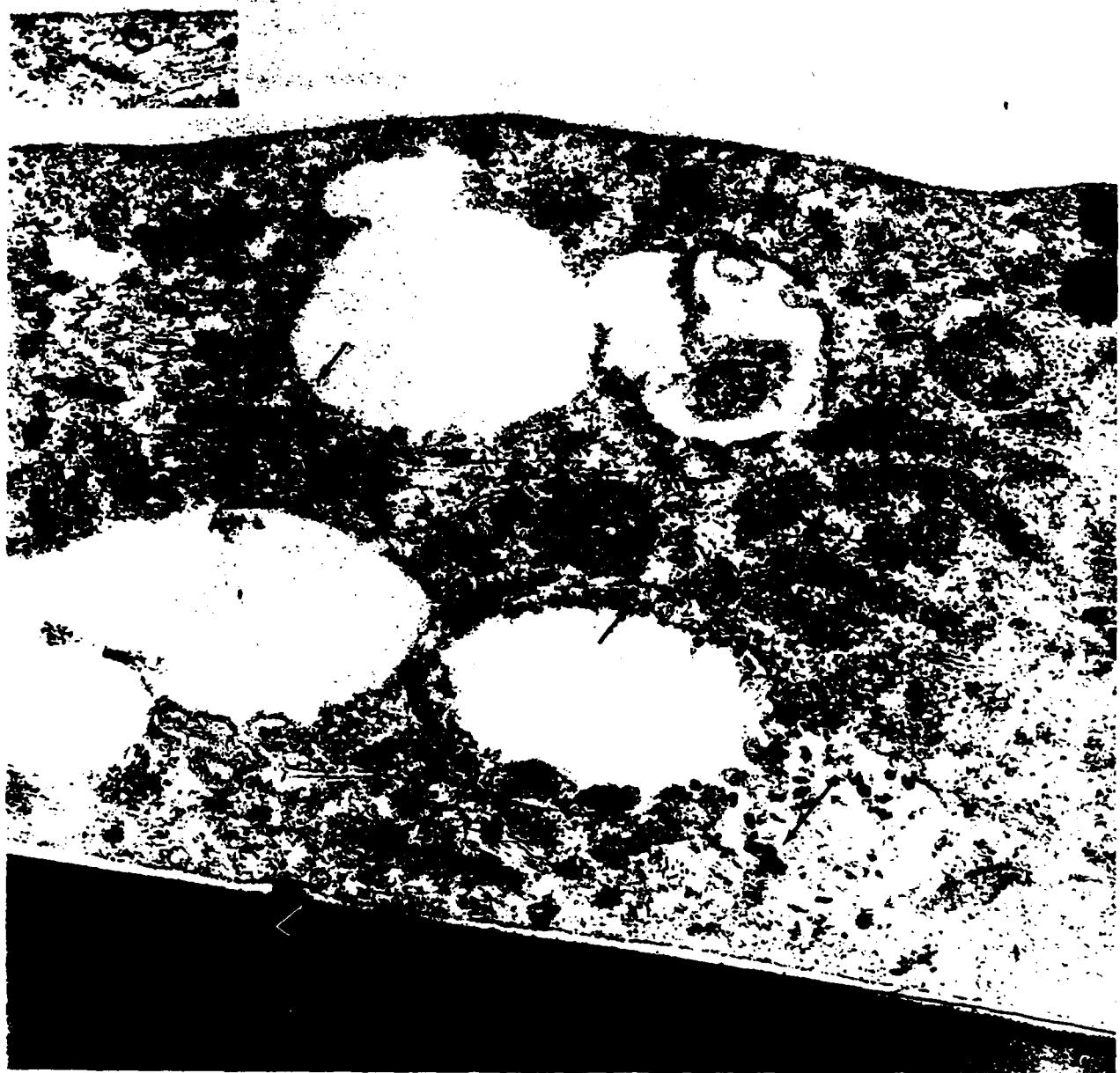


FIG. 9